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Tumor necrosis factor-alpha promoter variants and iron phenotypes in 785 Hemochromatosis and Iron Overload Screening (HEIRS) Study participants

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Abstract

We sought to determine if *TNF* promoter variants could explain iron phenotype heterogeneity in adults with previous *HFE* genotyping. HEIRS Study participants genotyped for C282Y and H63D were designated as high transferrin saturation (TS) and/or serum ferritin (SF) (high TS/SF), low TS/SF, or controls. We grouped 191 C282Y homozygotes as high TS/SF, low TS/SF, or controls, and 594 other participants by race/ethnicity as high TS/SF or controls. Using denaturing high-performance liquid chromatography (DHPLC), we screened the *TNF* promoter region in each participant. We performed multiple regression analyses in C282Y homozygotes using age, sex, HEIRS Study Field Center, and positivity for *TNF* −308G→A and −238G→A to determine if these attributes predicted ln TS or ln SF. DHPLC analyses were successful in 99.3% of 791 participants and detected 9 different variants; *TNF* −308G→A and −238G→A were the most prevalent. Most subjects positive for variants were heterozygous. The phenotype frequencies of each variant did not differ significantly ($p < 0.05$) across subgroups of C282Y homozygotes, or across white, black, Hispanic, and Asian non-C282Y homozygotes subgrouped as high TS/SF phenotypes and controls. *TNF* −308G→A positivity was a significant predictor of initial screening ln TS but not ln SF; *TNF* −238G→A predicted neither ln TS nor ln SF. We conclude that *TNF* promoter variants have little, if any, effect on initial screening SF values in adults with or without C282Y homozygosity. We cannot exclude a possible association of homozygosity for *TNF* promoter variants on TS and SF values.

Keywords

iron; iron overload; hemochromatosis; phenotype; tumor necrosis factor

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Introduction

Iron phenotype heterogeneity is incompletely explained by the common C282Y and H63D mutations of the hemochromatosis gene *HFE* on chromosome 6p21.3 [1]. It has been proposed that the proinflammatory cytokine tumor necrosis factor (TNF)-alpha encoded by *TNF* (OMIM *191160), ~250 kb centromeric of human leukocyte antigen (HLA)-B, is involved in the phenotypic expression of hemochromatosis [2, 3]. The *TNF* promoter variant −308G→A may increase expression of TNF-alpha [4-6]. A putative gene in linkage disequilibrium with *HFE* has also been reported to influence iron phenotypes in persons with *HFE* hemochromatosis [7-10]. It is possible that this gene is *TNF*, but no consistent association of iron phenotypes and the common *TNF* promoter variants −308G→A or −238G→A has been described [11-14].

Most previous studies of the effects of TNF alleles reported observations on European Caucasian subjects selected predominantly because they had hemochromatosis, iron overload, or hyperferritinemia, or in whom only *TNF* −308G→A was evaluated [11-14]. The present study participants were recruited from primary care populations, were found at screening to have diverse iron phenotypes, and had defined *HFE* C282Y and H63D genotypes. The present study includes observations on 191 *HFE* C282Y homozygotes, the second largest group of such subjects studied for TNF allele phenotypes and iron phenotypes to date [12]. Moreover, we evaluated the present participants for a larger number of *TNF* variants than was done in previous studies [11-14].

Denaturing high-performance liquid chromatography (DHPLC) is a reliable means of mutation screening that has been used to identify deleterious mutations in persons with hemochromatosis, iron overload, or hyperferritinemia [15-20]. We sought to identify *TNF* promoter variants that could explain iron phenotype heterogeneity in adults with previous *HFE* genotyping to detect C282Y and H63D. We used DHPLC to screen the *TNF* promoter region in each of 791 HEIRS Study participants. The possible influence of the mutations we detected on iron phenotypes in persons with and without C282Y homozygosity are discussed.

Methods

Study approval

The local Institutional Review Boards of the HEmochromatosis and IRon Overload Screening (HEIRS) Study Coordinating Center, Central Laboratory, and each Field Center approved the Study protocol that is described in detail elsewhere [21].The Field Centers recruited volunteer participants ≥25 years of age who gave informed consent [21].

Selection of participants for DHPLC analyses

The HEIRS Study screened 99,711 primary care participants 25 years of age or older [21, 22]. In this substudy, we used denaturing high-performance liquid chromatography (DHPLC) to identify mutations in the promoter region of *TNF*. We selected 75 *HFE* C282Y homozygotes with the highest transferrin saturation (TS) and serum ferritin (SF) values (high TS/SF), and 75 C282Y homozygotes with the lowest TS and SF (low TS/SF) [20, 23]. We randomly selected 76 C282Y homozygotes as controls; these included 16 high and 19 low TS/SF participants. We also selected 295 participants without C282Y homozygosity (74 non-Hispanic whites, 75 Hispanics, 74 blacks, 72 Asians) with the highest percentile for TS or SF of their respective race/ethnicity groups [23, 24]. We selected 299 other participants without regard for TS and SF as controls (75 non-Hispanic whites, 74 Hispanics, 75 blacks, 75 Asians) [23].

DHPLC screening was performed using a Transgenomic WAVE® 3500 HT system and a reverse-phase chromatography column (DNASep® HT) as described in detail elsewhere [23]. We used these PCR primers: AACCAGCATTATGAGTCTCCGGGT (forward) and GGGTGTGCCAACAACTGCCTTTAT (reverse). All samples that appeared to have a mutation were sequenced in both directions using the same PCR amplicons employed for screening.

We designated *TNF* promoter variants according to terminology used broadly in the literature. To permit consistency in reporting our results and comparing them with TNF-alpha promoter variants detected in other studies, we used the common variant −308G→A as the marker from which the location of other variants we discovered were determined. Mutations we detected include: −308G→A; −238G→A; −244G→A; −376G→A; −572A→C; −636G→T; −645G→A; and −77T→A.

Literature review

We performed a computerized review of the literature using http//www.pubmed.gov to identify studies in which −308G→A and −238G→A *TNF* promoter variant phenotype frequencies were reported in *HFE* C282Y homozygotes and in control subjects.

Statistical considerations

The original dataset consisted of data on 791 subjects; DHPLC analyses were unsuccessful in six of these (0.7%). Thus, there were evaluable observations on 785 subjects. There were 191 evaluable C282Y homozygotes; among them, 16 high TS/SF and 19 low TS/SF participants were also included in the control group. We grouped observations on 594 participants as either high TS/SF or controls, and by race/ethnicity. We performed power calculations to determine the probability of detecting significant differences (alpha 0.05) at allele frequency levels of 0.05 vs. 0.10 and 0.1 vs. 0.2, respectively, based on group sizes of 75 participants. The respective probabilities were 21% and 40%, respectively. TS and SF data are displayed in Tables as geometric means (95% CI); these values were computed as previously described in detail (25, 26]). Statistical analyses were performed using Saber (Statistical Analysis Battery for Epidemiological Research, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA; http://www.cdc.gov/nceh/publications/saber/saber.htm) and GB-Stat® (v. 10.0, 2003, Dynamic Microsystems, Inc., Silver Spring, MD). Descriptive data are displayed as enumerations or percentages.

Prevalences of *TNF* promoter variants were computed using data from participants whose DHPLC analyses were successful. Prevalences of *TNF* promoter variants in participant subgroups were compared using chi-square analysis or Fisher exact test, as appropriate. Using observations in the 191 *HFE* C282Y homozygotes, we performed multiple regression analyses to determine the effects of the independent variables (age, sex, Field Center, race/ethnicity (white vs. other), initial screening TS, and presence or absence of the −308G→A and −238G→A *TNF* promoter variants) on initial screening SF values measured at initial screening (dependent variable). Values of $p < 0.05$ were defined as significant.

Results

Participants with HFE C282Y homozygosity

TNF promoter variant positivity—Iron phenotypes and phenotype frequencies of *TNF* promoter variants in 191 *HFE* C282Y homozygotes are displayed in Table 1. The frequencies of participants positive for the *TNF* promoter variant −308G→A were greater than for other variants. The frequencies of participants positive for the respective variants

did not differ across high TS/SF, low TS/SF, and control groups of C282Y homozygotes (Table 1).

We also compared the phenotype frequencies of *TNF* promoter variants in high TS/SF C282Y homozygotes (Table 1) and in white controls without C282Y or H63D (Table 2). There was no significant difference between the groups for any of the *TNF* promoter variant phenotype frequencies (data not shown).

Determinants of serum iron measures in HFE C282Y homozygotes—We used age, sex, race/ethnicity (white vs. other), TS, Field Center, and *TNF* −308G→A and −238G→A positivity as independent variables and ln SF as the dependent variable to analyze data from 191 C282Y homozygotes. In multiple regression analyses, male sex and greater ln TS values were associated with significantly greater ln SF levels ($p = 0.0043$ and $p < 0.0001$, respectively). The presence of neither *TNF* −308G→A or −238G→A were significant predictors of initial screening ln SF levels ($p = 0.1443$ and $p = 0.9090$, respectively). When we used the same variables and employed ln SF as an independent variable and ln TS as the dependent variable, ln SF was a significant positive predictor of higher ln TS ($p < 0.0001$). Positivity for *TNF* −308G→A was a significant independent predictor of higher initial screening ln TS values ($p = 0.0463$). Positivity for −238G→A was not a significant predictor of ln TS ($p = 0.9251$).

Participants without HFE C282Y homozygosity

TNF −308G→A was the most prevalent variant we detected. Positivity for *TNF* −308G→A was greatest in whites and lowest in Asians (Table 2). Variants −77T→A, −572A→C, −636G→T, and −645G→A were uncommon in all race/ethnicity groups. When we compared prevalence rates for respective *TNF* variants between high TS/SF and control race/ethnicity groups, there were no significant differences. Positivity for −308G→A was lower in high TS/SF *HFE* C282Y homozygotes (Table 1) than in white controls (Table 2), but this difference was not significant (17.3% vs. 26.7%; $p = 0.1542$).

Previously reported −308G→A TNF and −238G→A promoter variant phenotype frequencies in different populations

We compiled phenotype frequencies of −308G→A and −238G→A *TNF* promoter variants in *HFE* C282Y homozygotes and control subjects in five studies (three in Europe, two in North America) (Table 3). This revealed that −308G→A frequencies were lower in North American subjects than in European subjects with *HFE* C282Y homozygosity, but these differences were not significant. Phenotype frequencies in control subjects were available in three studies. These frequencies did not differ significantly. The frequencies of −238G→A were higher in North American subjects than in European subjects with *HFE* C282Y homozygosity, but these differences were not significant. The frequency of −238G→A in U.S./Canada control subjects was lower than in Italian subjects but higher than in California subjects. These differences were not significant. In the three studies in which observations were available in *HFE* C282Y homozygotes and in non-hemochromatosis control subjects, the phenotype frequencies of *HFE* C282Y homozygotes and control subjects did not differ significantly Table 3).

Discussion

The present HEIRS DHPLC study has features not previously reported in earlier analyses of *TNF* promoter variants [11-14]. The present study includes observations on 191 *HFE* C282Y homozygotes, the second largest group of such subjects studied for TNF allele phenotypes and iron phenotypes to date [12], and includes participants with and without

abnormal iron phenotypes recruited from primary care populations. Most previous studies reported data on subjects selected predominantly because they had hemochromatosis, iron overload, or hyperferritinemia or evaluated only *TNF* −308 G→A [11-14]. Participants in the present study represented diverse racial/ethnic groups, whereas most of the previously reported subjects were European Caucasians. All participants in the present study had defined *HFE* C282Y and H63D genotypes, and included 191 *HFE* C282Y homozygotes representing diverse iron phenotypes detected at screening. The present analysis does not include observations on iron removed by phlebotomy to achieve iron depletion or on liver iron concentration, because obtaining these measures was beyond the scope of the HEIRS Study.

The phenotype frequencies of *TNF* promoter variant −308G→A do not differ significantly across *HFE* C282Y homozygotes studied in several geographic regions of Europe and North America [11, 12, 14, 27]. There is also no significant difference in the proportion of *HFE* C282Y homozygotes and non-hemochromatosis control subjects who were positive for this phenotype in these regions [11-14]. Except *TNF* −238A→G, the other *TNF* variants that we detected were uncommon.

In European control populations, the phenotype frequencies of *TNF* −238G→A were 7.3%-15.1% [11, 13]. We observed that phenotype frequencies of *TNF* −238G→A were 8.0% in *HFE* C282Y homozygotes with high TS/SF, and 12.0% in white controls without C282Y or H63D; these values did not differ significantly. In Swedish and Norwegian C282Y homozygotes and control subjects, no difference in the phenotypes frequencies of −238G→A was observed [13]. In contrast, the phenotype frequency of −238G→A was significantly lower in *HFE* C282Y homozygotes in Italy diagnosed to have hemochromatosis than in corresponding control subjects [11].

In the present study, the percentages of respective *TNF* variants in *HFE* C282Y homozygous participants did not differ across high TS/SF, low TS/SF, and control groups. This is consistent with results of a previous study that compared *TNF* variants in hemochromatosis patients with C282Y homozygosity and control subjects in Norway and Sweden [13]. In other reports, associations of *TNF* −308G→A or −238G→A with measures of iron overload or iron-related liver injury are conflicting [11, 12, 14]. In the present study, we did not observe differences in the phenotype frequency of any *TNF* promoter variant between high TS/SF and control participants without C282Y homozygosity of diverse races/ethnicities. Further, multiple regression analyses revealed that positivity for *TNF* −308G→A was not an independent predictor of either TS or SF in *HFE* C282Y homozygotes detected in population screening.

In vitro studies demonstrate that −308G→A is a stronger transcriptional activator than the corresponding wild-type allele (−308G) in phorbol myristate acetate-stimulated Jurkat and U937 cells [6] and in human B-cell lines [5]. Further, −308A induces production of greater quantities of TNF- alpha *in vitro* and *in vivo* than does −308G [28-30]. TNF-alpha inhibits iron transport in vitro through its action on divalent metal transporter-1 and ferroportin [31, 32], participates in the regulation of ferritin and transferrin receptor gene expression [33, 34], and is involved in the iron metabolism of macrophages [35, 36]. TNF-alpha does not act directly on injured hepatocytes to regulate hepcidin expression *in vitro* [32, 37, 38]. Taken together, it is predicted that increased expression of TNF-alpha decreases serum iron and TS levels and increases SF [35, 39, 40], although TNF-alpha does not act directly to induce hepcidin mRNA [41].

Monocytes and macrophages produce and release TNF-alpha. Gordeuk and colleagues reported that release of TNF-alpha by blood monocytes stimulated by lipopolysaccharide *in*

vitro was lower in hemochromatosis patients than in controls; the study was performed before the discovery of *HFE* in 1996, and genotyping to detect *TNF* variants was not reported in any of the subjects [2]. Fargion *et al.* reported that TNF-alpha release from blood monocytes in hemochromatosis patients without −308G→A was significantly lower than that in control with without −308G→A [11]. The same investigators reported that TNF-alpha release from blood monocytes hemochromatosis patients with −308G→A did not differ significantly from that of controls with −308G→A [11]. Distante and colleagues measured TNF-alpha release from blood mononuclear cells *in vitro* in a variety of subjects, and reported similar values in C282Y homozygotes with or without hemochromatosis phenotypes, persons with secondary iron overload, and control subjects grouped according to positivity or negativity for *TNF* −308G→A and −238G→A [13]. Taken together, these results suggest that an effect, if any, of hemochromatosis, *HFE* C282Y homozygosity, or TNF −308G→A on production and release of TNF-alpha by monocytes is small.

Most subjects with *TNF* −308G→A and–238G→A in the present study and in other reports involving iron metabolism were heterozygotes [11-14]. It is plausible that homozygosity for −308G→A or and −238G→A has a greater effect on iron phenotypes than heterozygosity. For example, there was a higher prevalence of iron deficiency anemia in West African children with −308A homozygosity than in those without this genotype [42]. Regardless, there are insufficient numbers of −308G→A homozygotes in the present and previous studies to permit meaningful independent analysis of observations in these participants [11-14]. Further, the phenotype frequencies of −308G→A, −238G→A, or other *TNF* promoter variants are too low to account for most serum iron phenotype heterogeneity in persons with or without *HFE* C282Y homozygosity. We conclude that *TNF* promoter variants have little, if any, effect on initial screening SF values in adults with or without C282Y homozygosity. We cannot exclude a possible association of homozygosity for *TNF* promoter variants on TS and SF values.

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Table 1

Iron phenotypes and phenotype frequencies of *TNF* promoter variants in 191 *HFE* C282Y homozygotes[a]

Characteristic	High TS/SF C282Y homozygotes (75)[b]	Low TS/SF C282Y homozygotes (75)[c]	Control C282Y homozygotes (76)[d]
Transferrin saturation, %	90 (86, 93)	36 (32, 41)	57 (50, 64)
Serum ferritin, μg/L	698 (553, 881)	78 (57, 106)	242 (175, 334)
−77T→A[e]	0	0	0.0132
−238G→A[e]	0.0800	0.0800	0.0394
−244G→A	0	0	0
−308G→A[f]	0.1733	0.1733	0.1711
−376G→A[e]	0.0133	0.0266	0
−572A→C[e]	0.0133	0	0.0132
−636G→T[e]	0	0.0133	0
−646G→A[e]	0.0133	0	0

[a] All data represent observations in *HFE* C282Y homozygotes. Transferrin saturation (TS) and serum ferritin (SF) data are displayed as geometric mean (95% CI); other data are expressed as *TNF* variant positivity (heterozygosity or homozygosity).

[b] There were 70 whites, 1 Hispanic, and 2 blacks; 1 participant reported unknown race/ethnicity, and 1 reported multiple race/ethnicity.

[c] There were 71 whites, 3 Hispanics, and 1 black.

[d] These 76 C282Y homozygotes included 16 high and 19 low TS/SF participants.

[e] All subjects were heterozygotes for these *TNF* variants.

[f] In high TS/SF participants, one subject was homozygous and 12 were heterozygotes for −308G→A; in low TS/SF participants, two subjects were homozygotes and 11 were heterozygotes for −308G→A.

Table 2

Iron phenotypes and phenotype frequencies of TNF promoter variants in HEIRS Study participants without HFE C282Y homozygosity[a, b]

Characteristic	Whites		Hispanics		Blacks		Asians	
	High TS/SF (74)	Controls (75)	High TS/SF (75)	Controls (74)	High TS/SF (74)	Controls (75)	High TS/SF (72)	Controls (75)
Transferrin saturation, %	69 (65, 74)	26 (23, 29)	61 (58, 64)	23 (21, 26)	74 (70, 79)	22 (20, 25)	68 (64, 72)	30 (27, 33)
Serum ferritin, μg/L	771 (642, 926)	97 (76, 103)	388 (299, 504)	73 (57, 94)	1,216 (1,021, 1,448)	95 (75, 121)	769 (652, 900)	163 (128, 208)
−77T→A	0.0135	0	0	0	0	0	0.0139	0
−238 G→A	0.0811	0.1200	0.0933	0.1216	0.0135	0.0667	0.0833	0.0800
−244G→A	0	0	0	0	0.1216	0.0533	0.0139	0
−308G→A	0.2703	0.2667	0.1200	0.1757	0.2568	0.1867	0.0972	0.0533
−376G→A	0.0135	0.0133	0.0267	0.0541	0	0.0133	0	0
−572A→C	0.0135	0.0133	0	0	0	0	0	0
−636G→T	0	0	0	0	0	0	0	0
−646G→A	0	0	0	0	0	0	0	0.0133

[a]Transferrin saturation (TS) and serum ferritin (SF) data are displayed as geometric mean (95% CI); other data are variant positivity (heterozygosity or homozygosity).

Table 3

Phenotype frequencies of two common *TNF* promoter variants in *HFE* C282Y homozygotes and control subjects

***TNF* variants, subjects**	**Italy [11]**	**California [12]**	**Sweden/Norway [13]**	**Germany [14]**	**U.S./Canada (present study)[a]**
***TNF* −308G→A**					
HFE C282Y homozygotes	0.2368 (9/38)	0.1296 (28/216)	0.2191 (39/178)	0.1977 (17/86)	0.1733 (13/75)
Population controls	0.2267 (39/172)	n.a.	0.2683 (11/41)	n.a.	0.2667 (20/75)
***TNF* −238 G→A**					
HFE C282Y homozygotes	0.0263 (1/38)	0.0278 (6/216)	0.0449 (8/178)	n.a.	0.0800 (6/75)
Population controls	0.1512 (26/172)	n.a.	0.0732 (3/41)	n.a.	0.1200 (9/75)

[a] In the present study, these respective subjects were *HFE* C282Y homozygotes with high TS/SF (see Table 1) and white control subjects who were wild-type (wt/wt) for *HFE* C282Y and H63D and who did not have high TS/SF (see Table 2).